Two novel viruses associated with severe disease symptoms of the green stinkbug *Nezara viridula*

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Two viruses were isolated from green stinkbugs (*Nezara viridula*) with severe disease symptoms. These viruses have been named *N. viridula* virus type 1 (NVV-1) and NVV-2 according to their relative sedimentation coefficients. NVV-1 is a small picorna-like virus with a diameter of 29 nm, a buoyant density in CsCl of 1.34 g/ml and a sedimentation coefficient of 153S. NVV-1 particles contain a 9.4 kb ssRNA segment and have three coat proteins of Mr 32 100, 31 500 and 30 7000. NVV-2 sediments as two components on sucrose gradients; the top 104S component consists almost entirely of 41 nm empty capsids and the faster sedimenting 177S component consists of intact 39 nm spherical particles. NVV-2 particles have a buoyant density in CsCl of 1.39 g/ml and consist of one major protein of Mr 73 800 and at least two minor proteins of Mr 13 500 and 16 500. Only one dsRNA segment of 6.2 kb was identified. The properties of NVV-2 are similar to those of the Totiviridae. Individual stinkbugs were infected with either NVV-1 or NVV-2, or with a mixture of the two viruses. Re-infection of virus-free stinkbugs with the mixture resulted in typical disease symptoms. Both viruses were vertically transmitted through the eggs and insects were infected by surface contamination of their food source.

In the subtropical regions of the Transvaal, South Africa, stinkbugs cause damage to a number of crops including macadamia (*Macadamia tetraphylla*), granadilla (*Passiflora edulis*) and avocado (*Persea gratissima*). They cause direct feeding damage to the fruit in addition to acting as vectors of several viruses that infect granadilla (von Wechmar & Brand, 1989). In the fruit and nut industry it is important to control stinkbugs until the end of the season, however for safety reasons farmers do not spray chemical insecticides for a certain period prior to harvesting. There is therefore an interest in alternative biocontrol agents.

While investigating virus transmission by the green stinkbug, *Nezara viridula* (Hemiptera: Pentatomidae), it was observed that stinkbugs originating from this subtropical region were more difficult to rear than insects collected in the Western Cape. In diseased colonies there appeared to be a reduction in the number of eggs that hatched and most of the insects did not develop past the fourth instar. Typical disease symptoms included apparent dehydration, fluid retention in the wings and a buffalo-type appearance whereby the thorax appeared abnormally large relative to the abdomen. Diseased stinkbugs also matured more slowly than did those in apparently healthy colonies (von Wechmar et al., 1991). Extracts of these insects were shown to contain two viruses, *N. viridula* virus type 1 (NVV-1) and NVV-2, which were not present in apparently healthy colonies. Reinfection of healthy colonies with mixed virus preparations of NVV-1 and NVV-2 resulted in typical disease symptoms.

Although there has been extensive investigation of insect pathogenic viruses, there have been no previous reports on viruses infecting stinkbugs. In this paper the characterization of two new viruses is reported: an ssRNA picorna-like virus and a monopartite dsRNA virus. The serological relatedness between these viruses and other insect viruses was investigated and a comparison of the properties of NVV-1 and NVV-2 with those of other viruses is made.

NVV-1 and NVV-2 were co-propagated in the green stinkbug, *N. viridula*. Stinkbug eggs coinfected with NVV-1 and NVV-2 originated from Nelspruit, South Africa and were supplied by I. J. Froneman (Citrus and Subtropical Fruit Research Institute, Nelspruit). Leaves containing attached eggs and first instar larvae were incubated in Petri dishes containing moist filter paper. Second and third instar insects were maintained in plastic containers with gauze lids (28 × 15 cm) containing green bean leaves (*Phaseolus vulgaris*), green beans, raw peanuts, sugar cubes and water-soaked cotton wool. Fourth instar to adult stinkbugs were reared on green bean, spinach (*Spinacia oleraceae*) or tomato plants (*Lycopersicon esculentum*) supplemented with green
Short communication

Fig. 1. Electron micrographs of sucrose density gradient-purified virions stained with uranyl acetate. (a) Empty NVV-2 particles; (b) NVV-1; (c) NVV-2. Bar marker represents 100 nm.

beans, peanuts, sugar cubes and water-soaked cotton wool and were maintained in gauze cages (50 × 50 × 50 cm). Cages were maintained under VHO growlux tubes with a light:dark cycle of 12 h:12 h (26 °C:20 °C). Insects were harvested when they reached adulthood or upon the development of severe disease symptoms.

Insects were ground in liquid nitrogen, homogenized in 0.1 M-potassium phosphate buffer pH 7.0, strained through cheesecloth and centrifuged at 10000 g for 10 min. The supernatant was centrifuged at 140000 g for 120 min and the viruses were separated by rate zonal density gradient centrifugation. Sucrose gradients (10 to 40 % in 0.1 M-phosphate buffer pH 7.0) were centrifuged at 27000 r.p.m. for 150 min in a Beckman SW28 rotor. Virus bands were located by light scattering and gradients were scanned at 254 nm and fractionated on an ISCO model 640 gradient fractionator coupled to an UA 5 absorbance monitor.

Virus preparations for electron microscopy were negatively stained with 2% uranyl acetate and were examined in a Zeiss EM109 transmission electron microscope. The size of the virus was measured directly from negatives using a Mitutoyo toolmaker's microscope.

Three opalescent bands were seen after sucrose gradient centrifugation. The top peak was composed almost entirely of 41 ± 2 nm empty capsids (10 determinations) (Fig. 1a). Although the second and the third peak were clearly resolved into two bands, u.v. absorption scans showed that there was contamination between the peaks. The majority of the particles in the second peak were spherical and 29 ± 1 nm in diameter (10 determinations) (Fig. 1b), whereas the majority of the particles in the third peak were spherical and had a diameter of 39 nm ± 1 (10 determinations) (Fig. 1c). The viruses isolated from peaks two and three will be referred to as NVV-1 and NVV-2, respectively. Spectrophotometric scans of particles in peak one had a maximum absorption at 280 nm and protein profiles were essentially the same as those in peak three (Fig. 2a), indicating that these particles were empty NVV-2 capsids. No external structural features were observed on any of these viruses. The use of organic solvents did not affect virus structure suggesting that neither virus had a lipid envelope.

The effect of pH was examined by purification in the following 0.1 ionic strength buffers: sodium acetate–NaCl pH 4 and 5; sodium phosphate–NaCl pH 6 and 7, sodium barbital–HCl–NaCl pH 8.0 (Miller & Golder, 1950) and by monitoring the relative yields after separation by sucrose gradient. NVV-1 was unstable at pH 4 or 5; good yields were obtained after purification at
pH 6 and 7 and lower yields at pH 8. NVV-2 was stable at all pH values tested with the best yields being obtained after purification at pH 6 or 7.

Sedimentation coefficients were calculated by rate zonal density gradient centrifugation using brome mosaic virus (Rybicki & von Wechmar, 1981), turnip yellow mosaic virus (Matthews, 1980) and aphid lethal paralysis virus (ALPV) (Williamson et al., 1988) as standards. Sedimentation coefficients of NVV-1, NVV-2 and NVV-2 empty capsids in 0-1 M-phosphate buffer were 153S ± 3S, 177S ± 3S and 104S ± 3S respectively (three determinations).

Buoyant densities were determined by isopycnic density gradient centrifugation. Virus samples were resuspended in a 1.377 g/ml cesium chloride in 0.1 M-density gradient centrifugation. Mr values of proteins were determined (Scotti, 1985). The buoyant densities of NVV-1 and NVV-2 were 1.34 ± 0.01 g/ml and 1.39 ± 0.01 g/ml respectively (four determinations).

Virus samples for protein analysis were purified by one cycle each of sucrose and caesium chloride density gradient centrifugation. M_r values of proteins were determined by SDS-PAGE on 10% and 12.5% polyacrylamide gels (Laemmli, 1970). Gels were stained with PAGE-blue 83 (BDH). NVV-1 preparations contained three major proteins of 32100 ± 400, 31500 ± 400 and 30700 ± 400 (five determinations) (Fig. 2a). These proteins were present in roughly equimolar ratios as assessed by the intensity of the bands after staining.

NVV-2 preparations contained one major protein and at least two minor proteins. The major protein had an M_r of 73800 ± 4100 (12 determinations) (Fig. 2a). The minor proteins had M_r of 16500 ± 700 and 13500 ± 400 (four determinations) and were detectable only if the sample was overloaded relative to the M_r 73800 protein (results not shown). Occasionally a protein of M_r 30000 was also visualized.

Nucleic acid was purified by SDS disruption and phenol–chloroform extraction (Brisco et al., 1985). The type and strandedness of the nucleic acid were determined by digestion with 0-1 M-protease K-treated DNase (Sigma) (Tullis & Rubin, 1980) in 20 mM-Tris–HCl pH 7.5, 10 mM-CaCl_2, 20 mM-MgCl_2 and with 10 μg/ml RNase A (Sigma) in 0.3 M NaCl and in water. After digestion, the nucleic acid was purified by phenol extraction. RNA size was estimated in 1% formaldehyde denaturing agarose gels (Sambrook et al., 1989).

NVV-1 nucleic acid was resistant to DNase digestion and susceptible to RNase A digestion in both high salt and water, indicating that it was ssRNA (results not shown). The RNA migrated as a single band when analysed by denaturing agarose gel electrophoresis and was 9.4 ± 0.1 kb (three determinations) in size (Fig. 2b). NVV-2 nucleic acid was resistant to DNase digestion and RNase A digestion in high salt, but was degraded by RNase A in water, indicating that it was dsRNA (results not shown). The thermal denaturation of NVV-2 RNA was measured in a Unicam SP1800 spectrophotometer. Nucleic acid samples were resuspended in 10 mM-NaCl, 20 mM-sodium acetate, 20 mM-sodium-cacodylate pH 8.0 and the change in u.v. absorbance (260 nm) was monitored with a rate temperature increase of 1 °C/min. NVV-2 RNA showed a single, sharp hypochromic shift with a T_m of 94.5 °C. This confirmed the double-stranded nature of the genome. A single band was seen after electrophoresis in 6-7, 0-9 and 1% agarose gels. An RNA size of 6-2 ± 0.1 kb (three determinations) was estimated (Fig. 2b). In order to denature the dsRNA effectively it was necessary to incubate it at 70 °C, as opposed to the recommended temperature of 65 °C (Sambrook et al., 1989).

The serological relatedness between viruses was initially assessed by indirect ELISA (Rybicki & von Wechmar, 1981). Microtitre plates were coated with 10 μg/ml sucrose gradient-purified NVV-1:NVV-2 (approximately 5:1) and probed with a fivefold antibody dilution series. Antisera to density gradient-purified NVV-1 and NVV-2 were raised in rabbits. Antiserum to the following viruses were acquired: Drosophila C virus (DCV) and cricket paralysis (CrPV) (N. F. Moore, Ministry of Defence, Whitelhall, London, U.K.), acute paralysis virus, black queen cell virus, Kashmir bee virus, sacbrood virus, bee slow paralysis virus and Sitobion avenae virus (M. Allen, Rothamsted Experimental Station, Harpenden, U.K.), tobacco necrosis virus (TNV) (von Wechmar et al., 1990), Rhopalosiphum padi virus (RhvPV) (Williamson et al., 1989) and ALPV (Williamson et al., 1988). No serological reaction was detected between NVV-1 and NVV-2 and antisera to the following viruses: CrPV, ALPV, RhvPV, bee slow paralysis virus, sacbrood virus, acute bee paralysis virus, black queen cell virus and TNV included as a negative control. Approximately twice background absorbances were obtained for the antisera to the following viruses: S. avenae virus, Kashmir bee virus and DCV.

In Western blots (Williamson et al., 1988) anti-NVV-1 sera (1/100 dilution) reacted specifically with three proteins of approximate M_r 30000. Anti-NVV-2 sera (1/100 dilution) reacted strongly with the 13500 M_r protein and to a lesser extent with the 73800 and 16500 M_r proteins (results not shown). Neither antiserum reacted with proteins in uninfected stinkbugs and antisera to TNV did not react with viral or stinkbug proteins. Kashmir bee virus and DCV antisera (1/100 dilution) did not react with NVV-1 or NVV-2 proteins in
Western blots. *S. avenae* virus antiserum (1/100 dilution) reacted with non-viral proteins indicating that the positive reaction obtained in indirect ELISA was not virus-specific.

The mode of virus transmission was investigated by feeding stinkbugs on raw peanuts which had been soaked in a virus suspension. Virus preparations were either purified by differential centrifugation (mixed virus preparation) or separated by sucrose and CsCl density gradient centrifugation. Virus-free stinkbugs fed on a mixed virus suspension became positive for both NVV-1 and/or NVV-2 when screened by double antibody sandwich (DAS)-ELISA (Rybicki & von Wechmar, 1981) 1 month after infecting. Infected stinkbugs became dehydrated in appearance or developed gross body abnormalities such as deformation of the wings or an abnormally large thorax relative to the abdomen. Repeated attempts to infect stinkbugs with only one virus were unsuccessful presumably owing to difficulties in obtaining biologically pure virus preparations.

Stinkbugs hatched from eggs from virus-infected colonies were infected with both viruses, indicating that both viruses were transmitted transovarially. Individual stinkbugs from several colonies were screened by DAS-ELISA and Western blotting for the incidence of infection. Stinkbugs were either infected with only NVV-1 or NVV-2, or were co-infected with both viruses.

In this study two previously undescribed viruses are characterized, NVV-1 and NVV-2. The properties of NVV-1 are similar to those of the *Picornaviridae* (Table 1). NVV-1 particles are small, icosahedral, are composed of three capsid proteins of approximate *M* of 30,000, contain ssRNA and have a density and sedimentation coefficient similar to those of other picornaviruses. The apparent absence of a fourth low *M* protein (VP4) and a high *M* precursor protein (VP0), typical of picornaviruses, could be due to the fact that these proteins are usually more difficult to detect (Williamson et al., 1989). It is possible that VP4 is lost during purification, as has been observed with other picornaviruses (Mak et al., 1970). Picorna- and picorna-like viruses have been found to infect a large range of insect taxonomic groups, including the Hemiptera (Moore et al., 1985). Detailed studies on the characterization and replication of some of these viruses have been done, including CrPV, DCV, infectious flacherie virus of silkworms (Moore et al., 1985) and the aphid viruses RhPV and ALPV (Williamson et al., 1988, Williamson & Rybicki, 1989). Unlike other insect picornaviruses such as RhPV (Williamson et al., 1989), CrPV (Eaton & Steacie, 1980) and DCV (Jousset et al., 1977), NVV-1 was acid-labile.

Although there was a weak serological reaction between intact virions and antiserum to DCV and Kashmir bee virus, no reaction was detected when disrupted virus was screened by Western blotting, indicating little or no serological relatedness. No such relatedness was detected using antisera against a number of other insect picorna- and picorna-like viruses. The serological distinctness of NVV-1, in addition to its physical properties, indicate that it is a unique virus and is probably a member of the *Picornaviridae*. Further characterization of the 3' and 5' RNA termini, elucidation of the replication strategy and determination of the nucleotide sequence of NVV-1 would be required to confirm its classification.

The second virus isolated, NVV-2, was unusual in that it contained only a single segment of dsRNA. The only other virus of higher eukaryotes with a similar RNA composition is heat-shock-resistant protein synthesis (HPS-1) virus isolated from a cultured *Drosophila melanogaster* cell line (Scott et al., 1980). NVV-2 is similar to HPS-1 virus in morphology, buoyant density, genome type and size but differs in protein profile. NVV-2 is stable over a wide pH range and has an unusually stable nucleic acid with a *Tm* of 94.5 °C.

The only viruses of higher eukaryotes to contain dsRNA are the reoviruses and the birnaviruses, both of which have more than one RNA segment. However, the *Totiviridae*, whose accepted members consist solely of mycoviruses, are monopartite dsRNA viruses (Francki
et al., 1991). The type member of the Totiviridae is the killer virus of Saccharomyces cerevisiae (Buck et al., 1984; Francki et al., 1991). In addition to genome type and size, the physicochemical properties of NVV-2 are markedly similar to those of the totiviruses (Table 1). It is possible that this family encompasses a broad virus host range as it was recently proposed that two proteozoan viruses, Giardia lamblia virus (GLV) (Furfine et al., 1989) and Trichomonas vaginalis virus (Wang & Wang, 1986) have possible affinities to this family (Francki et al., 1991). Microsporidia spores have been detected in stinkbug colonies (R. Klug, Plant Protection Institute, Cedara, Pietermaritzburg, South Africa, personal communication) and it would be of interest to investigate the site of virus multiplication and to determine whether proteozoan infecting stinkbugs are involved in virus production. The assignment of NVV-2 to the Totiviridae will require determination of its nucleotide sequence, genome organization and mode of gene expression.

The severe disease symptoms manifested in infected stinkbugs and the apparent highly contagious nature of the viruses indicate that they have potential as control agents. Analysis of samples collected over a 2 year period indicates that both viruses persist in the field, and it is possible that the viruses are in fact already limiting natural stinkbug populations.

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References


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